Comprehensive analysis of *RHD* alleles in Argentineans with variant D phenotypes

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BACKGROUND: The serologic assignment of the RhD status may be hindered in patients with weak D expression. A comprehensive study of *RHD* alleles occurring in the mixed population of Argentina is necessary to evaluate the most suitable DNA typing strategy.

STUDY DESIGN AND METHODS: A total of 18,379 patients from two stratified groups, Group 1 (G1; public hospital) and Group 2 (G2; private laboratory), were RhD phenotyped, and 88 samples with reduced D expression underwent molecular characterization.

RESULTS: The frequencies of D+, D–, and variant D phenotypes differed significantly ($p < 0.001$) between G1 and G2 (94.49% vs. 87.66%, 5.15% vs. 11.58%, and 0.36% vs. 0.75%, respectively). Eleven alleles were responsible for the weak D expression. Approximately 60% of the variant D phenotypes from G1 and G2 were weak D Types 1 through 4.0/4.2 and 25% were DVII. *RHD* alleles associated with African ancestry were encountered in G1. A new –282G>A mutation within the promoter region of *DAU-4* and *DOL* alleles was identified. Three weak D Type 1 samples on R_0 haplotypes were found in G1.

CONCLUSIONS: The D phenotype distribution in G2 resembles that in Europeans while the frequencies in G1 account for the Amerindian and African genetic contribution. The genotyping strategy described here is suitable to study D variants in the overall population and could allow a better use of the few available D– units and a rational administration of anti-D immunoprophylaxis. The results also show that *weak D Type 1* alleles do not exclusively segregate with a *Ce* allele, as assumed until present.

The Rh proteins are encoded by variant forms of the two highly homologous genes *RHD* and *RHCE*. The D antigen is by far the most clinically important because of its high immunogenicity and is still one of the leading causes of hemolytic disease of the newborn and posttransfusion alloimmunization.[1-3]

The serologic assignment of the RhD status may be hindered in individuals with a weak expression of the D antigen. It has been reported that among Europeans approximately 1% carry aberrant *RHD* alleles causing partial or weak D antigen expression.[4-6] These variant D phenotypes can only be distinguished by molecular studies and their characterization facilitates decision making on transfusion therapy, especially in countries like Argentina, facing a chronic shortage of D– red blood cell (RBC) units due to a rather low prevalence of D– phenotype. Moreover, D variant identification in pregnant women allows a more rational administration of RhIG prophylaxis.[7-10]

ABBREVIATIONS: G1 = Group 1; G2 = Group 2.

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Partial D and weak D types show substantial ethnic variability. Some alleles are confined to specific ethnic groups whereas others are more dispersed.[3,11-16] The population of Argentina is considered to be a mixture of white Caucasian Europeans, Amerindians, and Africans. In the main cities of the country, the European genetic component predominates as a consequence of the massive immigration from Spain and Italy at the beginning of the 20th century, whereas the Amerindian and African component reaches up to approximately 20 and 6%, respectively.[17-19] In contrast, the indigenous component is higher in the less developed northeastern and northwestern regions of the country. Previous observations suggest, however, that the Amerindian and African influence varies among different social groups within a city.[20-23] In this sense, a comprehensive study of the *RHD* polymorphism and allele distribution occurring in our population would help to determine the most suitable DNA typing scheme that could be used in blood bank services to appropriately classify D status.

In this study we have performed a survey of the *RHD* variants in Rosario, the third largest city of Argentina. We have been able to establish the population frequency of the variant D phenotypes in two stratified groups using a genotyping approach. The results obtained have also given some insight into the genetic variability of our population.

MATERIALS AND METHODS

Samples

Ethylenediaminetetraacetate blood samples were collected in consecutive order, during a 22-month time frame, with a written informed consent according to the Declaration of Helsinki. Two sample cohorts of unrelated adults of both sexes from the city of Rosario, the third largest city located in the central area of Argentina, were studied. The first cohort (Group 1 [G1]) included 12,672 samples collected at a public health care center, where the assistance is supported by the provincial government and is intended for the low-income population. The second cohort (Group 2 [G2]) included 5707 samples collected at a private clinical laboratory, where clinical tests are financed through insurance companies. None of the samples was selected or excluded on the basis of surname or additional blood group phenotype information. Each group represents different social strata of the population. It is estimated that approximately 48% of Argentineans have a social profile equivalent to G1 subjects while 52% is comparable to that of G2 subjects. Saline RBC suspensions were used for serologic studies and genomic DNA isolated from white blood cells by a modified salting-out method was used for molecular analysis.

Serologic analysis

The D antigen status of each sample was evaluated by hemagglutination with a commercially available monoclonal-monoclonal blended anti-D (Wiener Lab, Rosario, Argentina) containing immunoglobulin (Ig)M clone TH-28 and IgG clone MS-26, which reacts with most weak D and partial D RBCs, including DVI in the indirect antiglobulin test (IAT). When a weak agglutination reaction in saline of 3+ or weaker intensity was observed, the sample was further examined by incubation at 37°C for 15 minutes followed by recentrifugation. In addition to this, when the reagent showed an immediate spin-negative result, the RBCs were tested by the IAT. Samples with weak D antigen expression (1+ to 3+ agglutination intensity in saline or a positive IAT) were also tested with anti-C (Clone MS24), anti-c (Clone MS33), anti-E (Clone MS260), and anti-e (Clones MS16 + MS21 + MS63; DiaMed, Cressier sur Morat, Switzerland) and entered the work flow for molecular characterization. All standard serologic tests were performed in tube according to the manufacturer's instructions.

Molecular studies

Weak D type polymerase chain reactions

In a first step, samples were analyzed for the presence of Weak D Type 1 through 4 as described.[8] In presumably weak D Type 4 (602C>G) samples, the 48G>C (templated), 819G>A (untemplated), and 1025T>C (templated) polymorphisms were further investigated by polymerase chain reaction (PCR) with sequence-specific priming. Three separate PCR procedures, each containing the forward primers 48Cfor,[22] Ds6s,[9] and Ds7s[9] paired with reverse primers targeting the *RHD* polymorphism at position +106 in Intron 1 (Dint1rev, 5′-AGATGGGGGAATCTTTTTCCTT-3′), A819 (WD4819R 5′-CACGCCTCCTGCCAACACT-3′), and C1025 (DAR1025R 5′-AAGCACCAGCAGCACAA TGTAGG-3′) nucleotides, respectively, were developed to amplify a 245-bp product from *weak D Type 4.1*, a 131-bp product from *weak D Type 4.0* and *4.1*, and a 210-bp product from *weak D Type 4.2* alleles.

RHD exon scanning

In the absence of these alleles, *RHD* Exons 2 to 10 were amplified as described.[7] An additional oligonucleotide primer pair (48Gfor[22] and Dint1rev) was used for *RHD* Exon 1-specific reaction (245-bp product).

DVII PCR

Subsequently, the analysis of the 329T>C polymorphism was performed with the *RHD/C* Exon 2-specific forward primer D-2-201[7] and a reverse primer containing at its 3′ end the polymorphic nucleotide C329 (DVIIR

5′-CCACCATCCCAATACCTGAACG-3′). A 169-bp product was indicative of a *DVII* allele.

PCR conditions

These new amplifications were performed with approximately 50 ng of genomic DNA in a final volume of 10 µL containing 0.2 µmol/L of each primer (except for primers used for internal positive controls that were at 0.1 µmol/L), 0.2 mmol/L dNTPs, 1.5 mmol/L $MgCl_2$ (or 2 mmol/L for 48G>C PCR), and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI) in appropriate buffer. PCR procedures started with one cycle of denaturation at 94°C for 3 minutes and followed with 20 cycles of 10 seconds at 94°C, 1 minute at 65°C (48G>C PCR), 70°C (819G>A PCR), 71°C (1025T>C PCR), or 69°C (329T>C PCR) for annealing and extension and 10 cycles of 30 seconds at 94°C, 1 minute at 61°C (48G> PCR), 67°C (819G>A PCR), 65°C (1025T>C PCR), or 63°C (329T>C PCR) for annealing and 30 seconds at 72°C for extension. In all PCR procedures, a 434-bp PCR fragment from the human growth hormone locus was coamplified as a positive control.[7] PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide.

Extensive RHD genotyping with a DNA chip

Genetic analysis of eight samples was performed using a microarray-based DNA chip platform (BLOODchip, Progenika Biofarma, Vizcaya, Spain) that allows the identification of more than 100 *RHD* alleles.[24,25] Briefly, PCR-amplified *RHD* regions were fragmented, labeled with a fluorescence marker, and hybridized to probes attached to the surface of a glass slide. Fluorescence intensity was detected by a confocal scanner, quantified, and analyzed on proprietary software to determine the genotype and predicted phenotype.

Sequencing

Direct automated sequencing on PCR products of the 10 *RHD* exons, adjacent intronic sequences, and 5′ promoter region was performed in uncharacterized samples and in apparently weak D Type 1 variants associated with a R_0 haplotype.[6,9] The *RHD* 5′ promoter region was sequenced using the reverse primer 5′-CCTGTGTCCGTCTCTGA-3′ (positions –1 to –17) and the forward primer 5′-CCACATCTCCTTTCTCTTCTG-3′ (positions –663 to –643). Exonic regions involved in the DIVa, DVa, and weak D Type 59 polymorphisms were also sequenced.

Rhesus box analysis

RHD zygosity of the weak D Type 1 and weak D Type 3 samples with a CCee phenotype was evaluated by amplification of the downstream and hybrid *Rhesus box*es followed by digestion of the PCR products with endonuclease *Pst*I. An additional restriction site that is only present in the hybrid *Rhesus box* (G5275) produces a 564-bp fragment diagnostic for the *RHD* gene deletion.[26]

RESULTS

Rh phenotypes

A total of 18,379 individuals from two outpatient populations of the city of Rosario were RhD phenotyped over a 22-month period. Samples belonging to G1 (n = 12,672) were collected at a public hospital while those of G2 (n = 5707) were obtained from a private clinical laboratory. Results of the D typing are shown in Table 1. The frequencies of D+, D–, and variant D phenotypes differed significantly between both groups ($p < 0.0001$, chi-square test). The occurrence of D– individuals and variant D phenotypes was lower in G1 than in G2 while the frequency of D+ individuals was higher in G1 ($p < 0.0002$, compared by means of the z test).

Among the 88 samples with a weak D antigen expression, 62 (70.45%) were C+, eight (9.09%) were E+, three (3.41%) were C+ and E+, and 15 (17.05%) were C– and E–. The complete Rh phenotype is shown in Table 2 together with the molecular findings.

RHD alleles in variant D phenotypes

Molecular analysis showed that 81 of the 88 samples carried *RHD* alleles that differed from the consensus sequence. A schematic representation of the *RHD* genotyping strategy and the alleles found is represented in Fig. 1. Weak D type-specific PCRs and the exon scanning approach identified the allelic variant in 55 of the 88 samples with weak D antigen expression. To characterize the remaining D variants, eight random samples were subsequently analyzed with BLOODchip. Four partial D category VII, two hemizygous IVS5-38del4 (possible weak D Type 59, linkage often observed in Spanish weak D samples, unpublished observations), and two apparently normal D+ samples were found. These results prompted us to implement a DVII PCR with sequence-specific priming strategy to continue the molecular characterization. In total, 22 DVII variants were found. On the other hand, *RHD* Exon 8 sequence analysis confirmed the 1148T>C mutation associated with *weak D Type 59* allele in the suspected samples. The remaining uncharacterized

TABLE 1. D antigen expression in both groups analyzed*

Group	D+	Weak D expression	D–
1 (n = 12,672)	11,974 (94.49)	45 (0.36)	653 (5.15)
2 (n = 5,707)	5003 (87.66)	43 (0.75)	661 (11.58)

* Data are reported as number (%). Percentages may not add up exactly 100.00% because of rounding.

TABLE 2. *RHD* alleles and complete Rh phenotype found in samples with weak D antigen expression

RHD allele	Polymorphism detected	Number of samples		Phenotype frequency	Observed Rh phenotype
Weak D Type 1	809T>G (Exon 6)	21	G1: 11	1:1,152	Ccee (n = 7), CCee (n = 1), ccee (n = 3)
			G2: 10	1:571	Ccee (n = 10)
Weak D Type 2	1154G>C (Exon 9)	9	G1: 6	1:2,112	ccEe (n = 6)
			G2: 3	1:1,902	ccEe (n = 3)
Weak D Type 3	8C>G (Exon 1)	8	G1: 4	1:3,168	Ccee (n = 3), CCee (n = 1)
			G2: 4	1:1,427	Ccee (n = 4)
Weak D Type 4.0	602C>G (Exon 4), 819G>A (Exon 6)	11	G1: 2	1:6,366	ccee (n = 2)
			G2: 9	1:634	ccee (n = 9)
Weak D Type 4.2	602C>G (Exon 4), 1025T>C (Exon 7)	4	G1: 4	1:3,168	ccee (n = 4)
			G2: 0	0	
DVII	329T>C (Exon 2)	22	G1: 10	1:1,267	Ccee (n = 9), CcEe (n = 1)
			G2: 12	1:476	Ccee (n = 12)
Weak D Type 59	1148T>C (Exon 8)	2	G1: 1	1:12,672	Ccee (n = 1)
			G2: 1	1:5,707	Ccee (n = 1)
DVa	*RHD* exon scanning: Exon 5 negative. Mutations detected by sequencing: 667T>G, 697G>C, 712G>A, 733G>C, 744C>T, 787G>A, 800A>T (Exon 5)	1	G1: 1	1:12,672	Ccee (n = 1)
			G2: 0	0	
DIVa	*RHD* exon scanning: Exon 7 negative. Mutations detected by sequencing: 186G>T (Exon 2), 455A>C (Exon 3), 1048G>C (Exon 7)	1	G1: 1	1:12,672	Ccee (n = 1)
			G2: 0	0	
DAU-4	–282G>A (5′ promoter), 697G>A (Exon 5), 1136C>T (Exon 8)	1	G1: 1	1:12,672	CcEe (n = 1)
			G2: 0	0	
DOL	–282G>A (5′ promoter), 509T>C (Exon 4), 667T>G (Exon 5)	1	G1: 1	1:12,672	ccee (n = 1)
			G2: 0	0	
Consensus	Sequence of the 10 *RHD* exons and the promoter region	7	G1: 3	1:4,224	Ccee (n = 2), CcEe (n = 1)
			G2: 4	1:1,427	CCee (n = 4)

samples were fully sequenced. A *DAU-4* and a *DOL* allele were found. In these two samples, an additional G>A mutation at position –282 within the promoter region was also detected. In summary, 11 different alleles were found to be responsible for the weak expression of the D antigen. No alterations in the *RHD* exons, adjacent intronic sequences, and promoter region were detected in seven samples. The *RHD* alleles found are listed in Table 2. The cumulative frequencies in the overall population of the variant D phenotypes are also shown.

Table 3 shows the distribution of variant D phenotypes among samples with reduced D expression. Weak D Type 4.2, DIVa, DAU-4, and DOL variants were only encountered in G1 whereas weak D Type 4.0 was more frequently found in G2. Weak D Types 1, 2, and 3, for which no anti-D immunization event has been documented so far, represented together the 43.18% of the 88 variant D phenotypes. If partial D samples are not included in the analysis, weak D types are distributed as shown in Fig. 2. Among the weak D samples found in this study, weak D Types 1, 2, and 3 represent the 69.09%.

Taking into account that carriers of aberrant D phenotypes that show a weak D antigen expression (1+ to 3+ agglutination intensity in saline or a positive IAT) are considered D– for transfusion or pregnancy, we estimated the occurrence of such phenotypes among the 1402 individuals (1314 D– plus 88 D variant) that would receive D– RBCs or the immunoprophylaxis (Table 4). It was found that 1 of 37 patients that today are considered D– could in fact be managed as D+.

RHD sequencing of weak D Type 1 samples with a ccee phenotype

Analysis of the *RHD* exons, adjacent intronic sequences, and the promoter region of the three weak D Type 1 samples associated with a R_0 haplotype revealed a *RHD* allele that did not differ from the consensus sequence except for the 809T>G nucleotide substitution responsible for the *weak D Type 1* variant.

Rhesus box analysis

A hybrid *Rhesus box* was detected in both weak D Type 1 and weak D Type 3 samples with a CCee phenotype, suggesting that these samples were hemizygous for the corresponding *RHD* allelic variant with a r′ haplotype in trans.

DISCUSSION

The large number of variant D phenotypes described so far sometimes complicates the serologic assignment of a

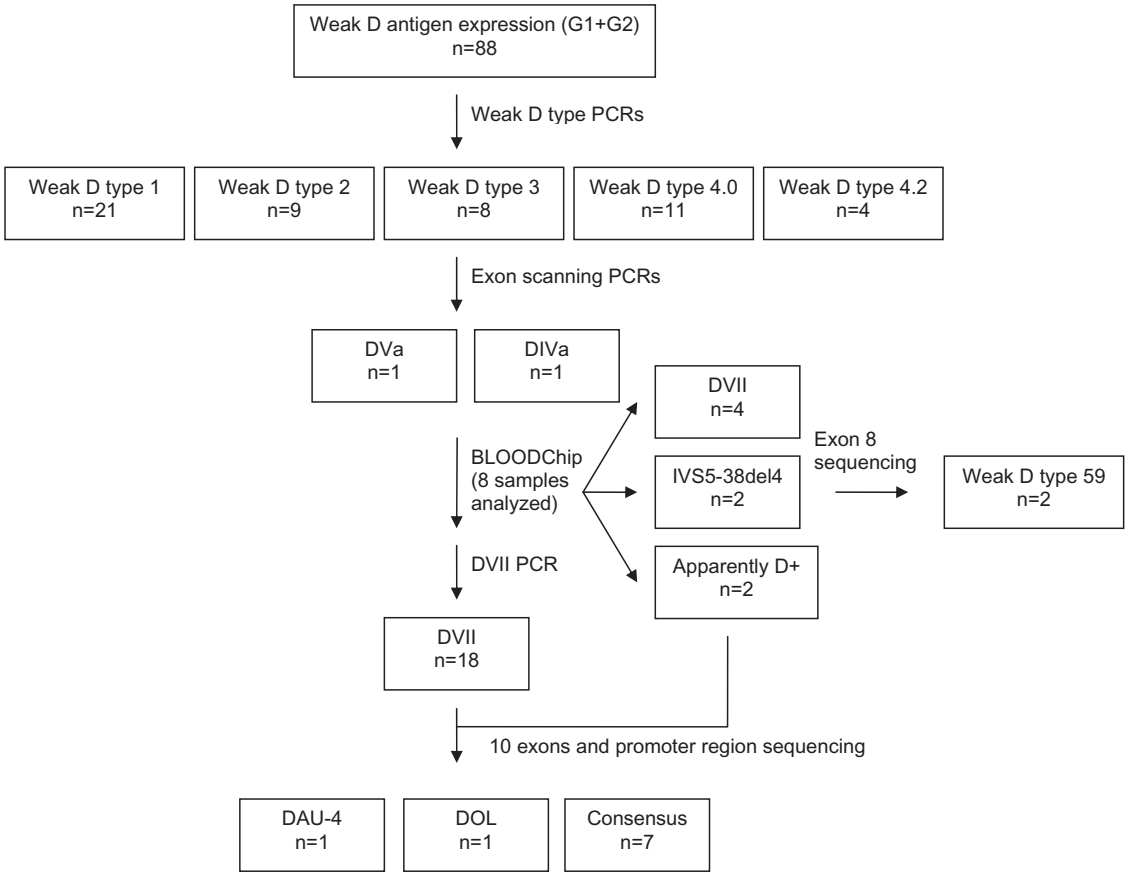

Fig. 1. *RHD* genotyping strategy and *RHD* alleles found in 88 samples with weak D antigen expression.

TABLE 3. Distribution of variant D phenotypes found in samples with weak D antigen expression*

Phenotype	G1 (n = 45)	Frequency (%)*	G2 (n = 43)	Frequency (%)*	G1 + G2 (n = 88)	Frequency (%)*
Weak D Type 1	11	24.44	10	23.26	21	23.86
Weak D Type 2	6	13.33	3	6.98	9	12.23
Weak D Type 3	4	8.89	4	9.30	8	9.09
Weak D Type 4.0	2	4.44	9	20.93	11	12.50
Weak D Type 4.2	4	8.89	0	0	4	4.55
DVII	10	22.22	12	27.91	22	25.00
Weak D Type 59	1	2.22	1	2.33	2	2.27
DVa	1	2.22	0	0	1	1.14
DIVa	1	2.22	0	0	1	1.14
DAU-4	1	2.22	0	0	1	1.14
DOL	1	2.22	0	0	1	1.14
Consensus	3	6.67	4	9.30	7	7.95

* Percentages may not add up exactly 100.00% because of rounding.

patient's D status and results in the wastage of D– units when they could have safely received D+ RBCs.[1,2,10] In this work, we performed a population study to examine the *RHD* alleles responsible for altered D antigen expression. Although Argentineans are considered to be a mixture of Europeans, Amerindians, and Africans, some evidence suggests that these different ethnic influences are not distributed homogeneously.[17-21,23] For this reason we studied blood samples from individuals who were stratified into two groups (G1 and G2) according to the type of health care system they have, which correlates with their social status. The large number of samples analyzed allowed us to determine that the distribution of D phenotypes differ significantly between both groups (Table 1). The percentages of D+, D–, and variant D phenotypes found in G2 resemble those reported for Europeans.[1,4-8,12-16,27] In contrast, the frequencies found in G1 may account for the Native American genetic contribution.[27] In addition, aber-

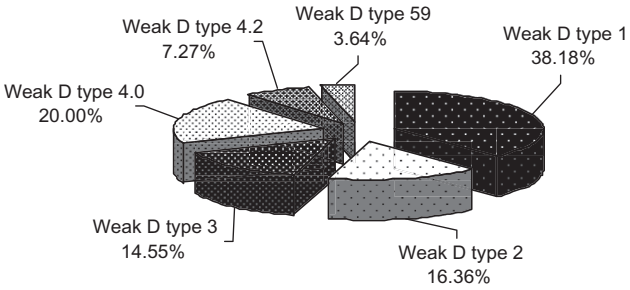

Fig. 2. Distribution of weak D phenotypes. Few molecular weak D types comprise the majority of all weak D alleles. Excluding partial D phenotypes, the frequencies of weak D Types 1, 2, 3, 4.0, and 4.2 found (96%) are comparable to those published for some European populations. However, only 6 different weak D alleles were encountered while the overall repertoire in Europeans seems to be much broader.[6,8,12-16]

TABLE 4. Frequency of variant D phenotypes among the total 1402* individuals currently considered D– for transfusion or pregnancy

Phenotype	Frequency
Weak D Type 1, 2, or 3 (n = 38)†	1:37
Other variant D (n = 50)	1:28

* 1402 corresponds to 1314 truly D– plus 88 variant D phenotypes.
† These phenotypes could be considered D+.

rant *RHD* alleles that are primarily associated with African ancestry[3,11] (*weak D Type 4.2*, *DIVa*, *DAU-4*, and *DOL*) were only encountered in G1, suggesting that the African influence is also more represented in this group. The low frequency of D– individuals in the overall Argentinean population (Table 1) together with the current replacement donor system make D– blood shortage a critical problem in blood bank facilities. The molecular identification of weak D Type 1, 2, or 3 carriers (43.18% of D variants, Table 3) may allow managing them as D+[10] and, thus, rationalize the use of D– stock units. Considering that 1 of 37 apparently D– patients carries a weak D Type 1, 2, or 3 (Table 4) and that every blood recipient is transfused, on average, 2 RBC units, the implementation of the weak D genotyping strategy may save more than 5% of D– units, which could be reserved for patients who are really at risk of anti-D alloimmunization. Moreover, the identification of weak D Type 1, 2, or 3 in pregnant women would prevent the administration of the anti-D immunoprophylaxis to these women, avoiding unnecessary costs and possible side effects.

This study also shows a surprisingly high frequency of D category VII among variant D phenotypes in Argentina (25.00%, Table 3). Although a frequent occurrence of DVII has already been observed in Caucasians from central Europe, in this study, the cumulative population frequency for D category VII was approximately 1 in 400 in G2, doubling the reported for some German populations.[5,28] The difference in occurrence may be attributed to the serologic method used in this work for the selection of samples with reduced D expression. It is known that almost all DVII variants are classified D+ with more sensitive serologic techniques and are not considered for molecular characterization. Nevertheless, there can also be substantial regional variations in the distribution of some partial D phenotypes that would explain these results. Even though the rate of alloimmunization in DVII patients is low,[29] the use of D– units, mainly in women of childbearing age and the administration of anti-D immunoprophylaxis would be advisable. Considering the pattern of reactivity of DVII samples and the lack of a routine anti-D reagent nonreactive with this D variant, the implementation of DVII genotyping on the examination of samples reacting 3+ or less in tube would detect DVII patients and prevent alloimmunization.

In this molecular screening, no DVI phenotype, one of the clinically most important partial D, was encountered among the samples with aberrant expression of the D antigen. This was also an unexpected result since the reported frequencies of D category VI for Europeans range from 1 in 2000 to 1 in 5000 and some authors have encountered up to 8% of DVI phenotypes among samples with serologically unclear D status. Indeed, *DVI* alleles have been described in the Spanish population, with a specific molecular background (*DVI Type 4*) being prevalent in their DVI population.[4,15,30-32] The admixture events that took place at the beginning of the 20th century mainly between Spanish and Italian immigrants with the local population probably diminished the frequency of this partial D variant in the Argentinean population.

In this study we found two samples carrying the previously described alleles *DAU-4* and *DOL*. However, in both of them, an additional templated G>A mutation was found at the D-specific position –282 within the promoter region. This D to CE change had not been previously reported. Even though the polymorphisms in the coding region of these alleles are responsible for the weak intensity reactions observed, alterations in the promoter sequence may contribute to the lower expression of the encoded protein. Molecular variants of the *RHCE* gene promoter have been associated with aberrant CE phenotypes in samples where no changes in the coding sequence were found.[33] Further studies are necessary to establish the participation of sequence polymorphisms in the promoter region of variant D alleles responsible for a weak D antigen expression.

In seven other samples, analysis of the entire *RHD* coding sequence predicted an unaltered protein and mutations in the promoter region were neither found. The low D antigen reactivity of these samples could be attrib-

uted to the diminishing effect of the C antigen, which was coexpressed in all of them.[34]

Interestingly, we also found up to three weak D Type 1 samples with a ccee phenotype, which did not correlate with the previously reported weak D Type 1-associated *RH* haplotype.[6] *RHD* sequencing analysis of these samples did not reveal any additional mutation and confirmed the *weak D Type 1* allele, suggesting that this D variant do not exclusively segregate with a *Ce* allele (R_1 haplotype), as assumed until present. This finding is consistent with a previous study that revealed an increased incidence of R_0 haplotypes in the Argentinean population attributed to African ancestry.[23] It is worth noting that these samples were encountered in individuals of G1 where the African contribution to the *RHD* allele pool is more evident (Table 3). The finding of *weak D Type 1* on R_0 haplotypes has never been described previously and could be the result of genetic recombination events occurring in an admixed population.

In this study, a large number of samples has been analyzed and the results show the incidence of aberrant *RHD* alleles in our population. We have also demonstrated that the use of the standard PCR procedures described here would identify more than 87% (77/88) of the samples with weak D antigen expression in the Argentinean population, which could help for clinical decision making in transfusion and obstetric medicine. This approach would allow a better use of the few available D– units and a rational administration of anti-D immunoprophylaxis without adding much to the cost of D typing.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to the manuscript submitted to **TRANSFUSION**.

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